Remarks

Claims 16-17 and 20-36 are cancelled. Claims 1-15, 18-19 and 37-59 are pending and subject to a Restriction Requirement. Applicants believe no new matter is added herein. A formal request for an interview to discuss the restriction requirement is submitted herewith. Reconsideration of the application is respectfully requested.

Restriction Requirement

In response to the restriction requirement, Applicants elect Group II (claims 37-49), drawn to a method of enhancing the immunogenicity of a vaccine and the species *Bacillus anthracis* (anthrax) with traverse.

It is demonstrated in the present application that immunostimulatory CpG ODNs can be used to induce an immune response to agents involved in bioterrorism. This immune response can be used to treat an infection with a bioterrorism agent, or it can be used preventively, such as to enhance the efficacy of a vaccine. Methods to enhance an immune treat an infection with a bioterrorism agent, and methods of enhancing the immunogenicity of a vaccine both include the administration of an antigen from the bioterrorism agent in combination with an immunostimmulatory CpG ODN to produce an immune response in a subject. Thus, Applicants submit that it would not be an undue burden on the Examiner to examine the claims of Groups I and II together.

Baccillus anthracis is an aerobic gram positive spore-forming bacturum. Anthrax Vaccnie Adsorbed (AVA) is a vaccine against *Bacillus anthracis* that is approved for use in the United States. Protective antigen (PA) is the major immunogen of the anthrax vaccine. Antibodies against PA neutralize the toxin. It is disclosed in the specification that immunostimulatory CpG ODN accelerate and boost the protective immunity elicited by a vaccine to *Bacillus anthracis.* In support of the specification, submitted herewith as Exhibit A is Klinman et al. (Vaccine 22: 2881-6, 2004) and Xie et al. (Infection and Immunity 73: 828-833, 2003) documenting the unexpectedly superior results obtained with immunostimulatory CpG ODN and a vaccine to *bacillus anthracis.*

The Office action further requires the election of a single species of oligodeoxynucleotide for initiation prosecution. Applicants' called Examiner Le on November 5, 2007 to discuss this requirement to elect only a single oligodeoxynucleotide. Specifically, a combination of three

oligodeoxynucleotides (SEQ ID NO: 23, SEQ ID NO: 24, and SEQ ID NO: 25) is particularly efficacious, and the Applicants would like to know if a specific combination of three ODN can be elected. In addition, ODN7909 (which comprises SEQ ID NO: 31) is also efficacious.

After waiting three business days the Applicants' represented again tried to contact the Examiner on November 9, 2007. However, the undersigned was unable to contact the Examiner. Thus, the Applicants were unable to determine if the Examiner would consider examining the combination of three ODNs.

Thus, in response to the requirement for an election of species, Applicants elect ODN7909, which comprises SEQ ID NO: 31. Applicants would like to elect ODN7909 itself.

Applicants would very much appreciate if Examiner Le and Examiner Campell would contact the undersigned for a telephone interview, prior to substantive examination, to allow them the opportunity to discuss ODN7909, and to determine if a combination of ODNs (SEQ ID NO: 23, SEQ ID NO: 24 and SEQ ID NO: 25, in specific combination) can be examined in the present application, and prior to making the restriction requirement final. A specific written request for an interview is provided below.

The claims are directed to a method of enhancing the immogenicity of a vaccine (claim 37), which can be to *Bacillus anthracis* (claims 39, 40 and 57). ODN7909 (comprising SEQ ID NO: 31) is an immunostimulatory K ODN (claim 51). The vaccine can be any type of vaccine, including but not limited to, antigen vaccines, attenuated vaccine, or heat killed vaccines (claim 38). The vaccine can be anthrax vaccine attenuated (AVA), and thus can include protective antigen (claims 41-42 and 58-59). ODN7909 comprises SEQ ID NO: 31 (claim 51). The immunostimulatory CpG ODN can be administered before, concurrently with or after a vaccine (claims 51-56).

Written Request for Interview

The Examiner is formally requested to contact the undersigned prior to issuance of the next Office Action in order to arrange a telephonic interview to discuss the requirement for an election of species. Applicants would like the opportunity to discuss the requirement to elect a single oligodeoxynucleotide sequence for initial examination. This request is being submitted under MPEP § 713.01, which indicates that an interview will be arranged in advance by a written request.

Respectfully submitted,

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CpG Oligodeoxynucleotides Adsorbed onto Polylactide-Co-Glycolide Microparticles Improve the Immunogenicity and Protective Activity of the Licensed Anthrax Vaccine

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To reduce the biothreat posed by anthrax, efforts are under way to improve the protection afforded by vaccination. This work examines the ability of immunostimulatory CpG oligodeoxynucleotides (ODN) adsorbed onto cationic polylactide-co-glycolide (PLG) microparticles (CpG ODN-PLG) to accelerate and boost the protective immunity elicited by Anthrax Vaccine Adsorbed (AVA, the licensed human anthrax vaccine). The results indicate that coadministering CpG ODN-PLG with AVA induces a stronger and faster immunoglobulin G response against the protective antigen of anthrax than AVA alone. Immunized mice were protected from lethal anthrax challenge within 1 week of vaccination with CpG ODN-PLG plus AVA, with the level of protection correlating with serum immunoglobulin G anti-protective antigen titers.

Bacillus anthracis is an aerobic gram-positive spore-forming bacterium found naturally in wild and domesticated animals (13). It is highly resistant to environmental degradation and produces a tripartite toxin that reduces the ability of the host's immune system to eliminate the pathogen (13). Human exposure to anthrax typically arises following contact with infected livestock and generally results in a mild form of cutaneous disease (8, 32). However, anthrax spores designed for aerosol delivery were intentionally released by bioterrorists in the United States in 2001. The resultant morbidity, mortality, and widespread panic underscored the potential for anthrax to be used as a bioterror agent and the need to improve the speed, magnitude, and safety of anthrax vaccination (25).

Anthrax Vaccine Adsorbed (AVA) is the only anthrax vaccine licensed for human use in the United States. It is prepared by adsorbing the culture filtrate of an attenuated toxinogenic nonencapsulated strain of *B. anthracis* (V770-NP1-R) onto aluminum hydroxide (16). Studies show that protective antigen (PA), the core of anthrax toxin, is the major immunogen of AVA. Antibodies against PA neutralize the toxin, inhibit spore germination, and improve the phagocytosis and killing of spores by macrophages (18, 26, 41, 42). Vaccination with AVA requires a series of six immunizations delivered over 18 months followed by yearly boosters (30, 31). This schedule has been linked to the development of adverse side effects including joint pain, gastrointestinal disorders, and pneumonia, leading many U.S. soldiers to refuse vaccination (9, 30, 33). Strategies designed to reduce the dose and number of immunizations required to achieve protection might improve compliance.

Synthetic oligodeoxynucleotides (ODN) containing immunostimulatory CpG motifs can boost the immune response to coadministered antigens, including AVA (4, 19, 20, 21, 40). CpG ODN induce the functional maturation of professional antigen-presenting cells and trigger the production of immunostimulatory cytokines and chemokines (2, 12, 20, 23, 24). Biodegradable cationic polylactide-co-glycolide (PLG) microparticles represent a different form of immune adjuvant. PLG improve the uptake and processing of adsorbed antigen by antigen-presenting cells (3, 6, 7, 28, 35, 36, 37). The current work examines whether CpG ODN adsorbed onto PLG microparticles (CpG ODN-PLG) increase the speed and magnitude of protective anti-PA immunity induced by coadministered AVA.

MATERIALS AND METHODS

Reagents. Phosphorothioate CpG ODN 1555 (GCTAGA*CG*TTAGCGT) and control ODN 1612 (GCTAGA*GC*TTAGCGT) were synthesized at the Center for Biologics Evaluation Research core facility (10). All ODN were free of endotoxin and protein contamination. ODN were adsorbed onto PLG at 1% (wt/wt) as previously described (37). Briefly, PLG microparticles with a copolymer ratio of 50/50 were emulsified with hexadecyl trimethyl ammonium bromide through a solvent evaporation process. The resultant cationic PLG microparticles were incubated with ODN overnight at 4°C with gentle shaking followed by washing and freeze-drying, and the amount of ODN adsorbed to PLG microparticles was quantitated. AVA was obtained from BioPort Corporation (East Lansing, Mich.). Recombinant PA was provided by U.S. Army Medical Research Institute of Infectious Diseases (Fort Detrick, Md.) and prepared as described (15). Recombinant lethal factor was purchased from Research Diagnostics Inc. (Flanders, N.J.). Toxinogenic (pXO1$^+$), nonencapsulated (pXO2$^-$) Sterne vaccine strain spores of *B. anthracis* (STI) were obtained from the U.S. Army Medical Research Institute of Infectious Diseases and stored at 4°C (17).

Animals. Specific-pathogen-free male A/J mice were obtained from the National Cancer Institute (Frederick, Md.). They were housed in sterile microisolator cages in a barrier environment and studied at 8 to 12 weeks of age. All animal experiments were conducted with Animal Care and Use Committee-approved protocols, and challenge studies were performed in a biosafety level 2 facility.

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EXHIBIT A

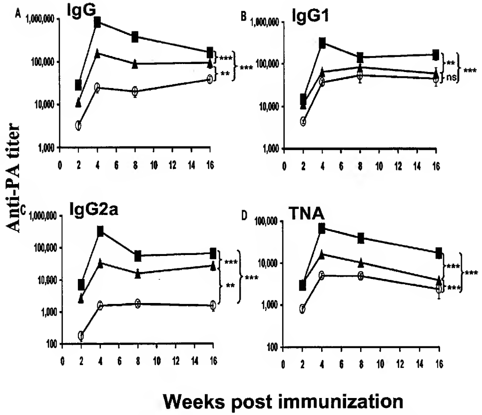

FIG. 1. IgG anti-PA antibody titers in AVA-vaccinated mice. Male A/J mice were immunized intraperitoneally with 200 μl of AVA (O) with and without 20 μg of free (▲) or PLG-adsorbed CpG ODN (■). Data represent the geometric mean ± standard error serum IgG anti-PA titer of 10 independently studied mice per group. **, $P < 0.01$; ***, $P < 0.001$; ns = not significant; determined by two-way analysis of variance. TNA, toxin-neutralizing activity.

Immunization and challenge studies. A/J mice were immunized intraperitoneally with AVA formulated in alum with and without CpG ODN, PLG, or CpG ODN adsorbed onto PLG (CpG ODN-PLG). The mice were bled weekly, and their serum was stored at −20°C until use. Mice were challenged intraperitoneally with 3×10^2 to 9×10^3 50% lethal doses (LD_{50}) of STI spores suspended in 0.5 ml of sterile phosphate-buffered saline (1 LD_{50} = 1.1×10^3 STI spores). Survival was monitored for 21 days.

IgG anti-PA ELISA. Immunoglobulin G (IgG) anti-PA antibody titers were monitored as described (21). Briefly, 96-well microtiter plates (Immulon 1B, Thermo Labsystems, Franklin, Mass.) were coated with 1 μg of recombinant PA per ml in phosphate-buffered saline at 4°C overnight. The plates then were blocked with 5% nonfat dry milk in phosphate-buffered saline containing 0.1% Tween 20. Plates were washed, and overlaid with serially diluted serum for 2 h at room temperature. After thorough washing, bound antibodies were detected by adding horseradish peroxidase-labeled goat anti-mouse IgG, IgG1, or IgG2a (Southern Biotechnology, Birmingham, Ala.) followed by ABTS (2,2'-azino-di-3-ethylbenzthiazoline-6-sulfonic acid) substrate (Kirkegaard & Perry, Gaithersburg, Md.). Relative antibody titers were determined by comparison to a standard curve generated with pooled sera from hyperimmunized mice and expressed as the reciprocal of the endpoint dilution which yielded an absorbance value at least three times background levels. All samples were analyzed in duplicate.

Toxin-neutralizing assay. The toxin-neutralizing titers of individual serum samples were assessed by their ability to protect RAW264.7 cells (American Type Culture Collection, Manassas, Va.) from lethal toxin with minor modifications from previously described methods (15). RAW264.7 cells were plated at 3×10^4 cells/well in 100 μl of glutamine-free RPMI 1640 medium containing 10% fetal bovine serum and 2 mM glutamax-1 (Invitrogen Corporation). The cells were incubated at 37°C in a 5% CO_2 incubator overnight. Serially diluted antiserum was 1:1 (vol/vol) mixed with lethal toxin (100 ng of recombinant PA per ml plus 100 ng of recombinant lethal factor per ml) at room temperature for 30 min to allow neutralization to occur; 100 μl of this mixture was then incubated with the cells for 6 h at 37°C. Cell viability was determined by monitoring the reduction of 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; Sigma-Aldrich, St. Louis, Mo.). Results were standardized against known high-titered monkey serum kindly provided by the Centers for Disease Control.

Statistics. Differences in the kinetic development of anti-PA immune responses were determined by two-way analysis of variance. Differences in the IgG anti-PA response induced by various vaccine-adjuvant combinations were assessed by one-way analysis of variance. Differences in survival were evaluated with chi-square analysis of Kaplan-Meier curves. Correlation coefficients were determined by linear regression analysis. The predictive value of IgG anti-PA and toxin-neutralizing titers on survival was evaluated with two-parameter logistic regression (1).

RESULTS

CpG ODN-PLG boost the immunogenicity of AVA. Previous studies established that CpG ODN could act as immune adjuvants when coadministered with AVA (21). To examine whether CpG ODN adsorbed onto PLG microparticles constituted an even more effective adjuvant, CpG ODN-PLG were coadministered to A/J mice with an optimally immunogenic dose of AVA (200 μl). A/J mice were selected for study because they are susceptible to challenge by attenuated STI anthrax spores, allowing the protective activity of the resultant

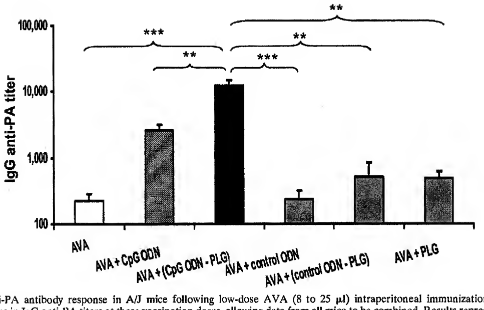

FIG. 2. IgG anti-PA antibody response in A/J mice following low-dose AVA (8 to 25 μl) intraperitoneal immunization. There were no significant differences in IgG anti-PA titers at these vaccination doses, allowing data from all mice to be combined. Results represent the geometric mean ± standard error IgG anti-PA response 14 days after immunization (n = 11 to 29 independently studied mice per group). **, $P < 0.01$; ***, $P < 0.001$, determined by one-way analysis of variance.

immune response to be examined in a biosafety level 2 facility (21).

Consistent with previous studies, the magnitude of the IgG anti-PA response induced by AVA was significantly improved by coadministration of CpG ODN ($P < 0.01$, Fig. 1A). However, coadministering CpG ODN-PLG with AVA boosted this response by an additional 4- to 30-fold ($P < 0.001$, Fig. 1A). This improved humoral immune response persisted for the duration of the study (4 months). IgG1, IgG2a, and serum toxin-neutralizing antibody titers were all significantly increased by combining CpG ODN-PLG with AVA (Fig. 1B to D). IgG anti-PA antibody was undetectable in unvaccinated mice.

Antigen-sparing effect of CpG ODN-PLG. The reactogenicity of the licensed anthrax vaccine might be reduced if the amount of AVA required to induce protective immunity could be lowered. Thus, the ability of CpG ODN-PLG to reduce the dose of AVA needed to elicit protective immunity was examined. Preliminary experiments established that 8 to 25 μl of AVA induced a detectable anti-PA response in all vaccinated mice, whereas 3 μl of AVA was immunogenic in only a fraction of vaccinated animals. As seen in Fig. 2, coadministering CpG ODN-PLG with 8 to 25 μl of AVA boosted the resultant IgG anti-PA antibody response by nearly 50-fold compared to AVA alone ($P < 0.001$, Fig. 2). This effect required the combination of CpG ODN with PLG, since PLG microparticles (alone or in combination with control ODN) had no significant impact on the magnitude of the response induced by AVA (Fig. 2).

CpG ODN-PLG accelerate the development of AVA-mediated protection. Preliminary studies demonstrated that mice immunized with 3 to 8 μl of AVA took >2 weeks to develop a protective immune response against anthrax infection (data not shown). To determine whether CpG ODN-PLG could accelerate this induction of protective immunity, A/J mice were immunized with AVA with and without adjuvant and their ability to resist anthrax challenge 1 week later was examined.

Consistent with preliminary studies, mice immunized with AVA alone were highly susceptible to infection at this early time point (>90% mortality, Fig. 3). Yet >80% of the mice immunized with CpG ODN-PLG plus AVA survived infection at this early time point ($P < 0.0001$, Fig. 3). Immunization with

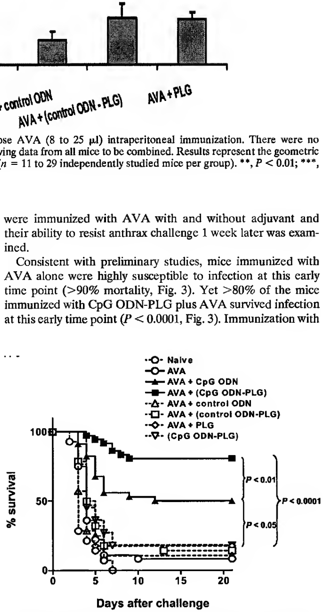

FIG. 3. Survival of vaccinated mice. A/J mice were immunized intraperitoneally with ≤8 μl of AVA plus 20 μg of free or PLG-adsorbed ODN. The mice were challenged intraperitoneally 7 days later with 3 × 10^{2-3} 50% lethal doses of STI spores. The survival of control groups (including naive mice and mice vaccinated with AVA) was indistinguishable between experiments. Thus, data from multiple experiments were combined for 11 to 36 mice per group.

CpG ODN plus AVA in the absence of PLG yielded intermediate protection (50% survival, $P < 0.01$ versus CpG ODN-PLG/AVA, Fig. 3), whereas CpG ODN-PLG in the absence of AVA was not protective.

Humoral immunity as a predictor of protection. There is considerable interest in identifying a surrogate marker for protective immunity against anthrax. Towards that end, the serum toxin-neutralizing activity and IgG anti-PA titer were evaluated as predictors of survival following anthrax spore challenge. As seen in Fig. 4A, toxin-neutralizing antibody correlated significantly with IgG anti-PA titer ($R^2 = 0.46$, $P < 0.0001$). Although toxin-neutralizing antibody predicted protection against anthrax, two-parameter logistic regression modeling showed that IgG anti-PA was the superior surrogate marker of survival (Fig. 4B, IgG: $R^2 = 0.64$, $P < 0.0001$; Fig. 4C, toxin-neutralizing antibody: $R^2 = 0.36$, $P < 0.0001$). In this context, receiver operating characteristic analysis showed that total IgG anti-PA titer was 97% accurate at predicting survival following anthrax challenge, whereas toxin-neutralizing antibody was 91% accurate. The magnitude of the IgG anti-PA response provided valuable information on an animal's resistance to anthrax infection. For example, >90% of mice are protected against 9×10^3 50% lethal doses of anthrax if their IgG anti-PA titer exceeds 1,000, while a titer of >6,000 indicates that >99% of mice are protected from such high-dose challenge.

DISCUSSION

Efforts are under way to increase the speed and magnitude of the protective immune response elicited by AVA. Current findings indicate that these goals may be met by coadministering CpG ODN-PLG with AVA, resulting in a more rapid and stronger anti-PA antibody response than immunization with AVA alone (or combined with CpG ODN in the absence of PLG; Fig. 1 and 2). Significant protection against anthrax challenge was present within 1 week of vaccination with CpG ODN-PLG plus AVA (Fig. 3), indicating that the combination of CpG ODN with PLG is significantly more effective than CpG ODN or PLG alone as an immune adjuvant. The quality

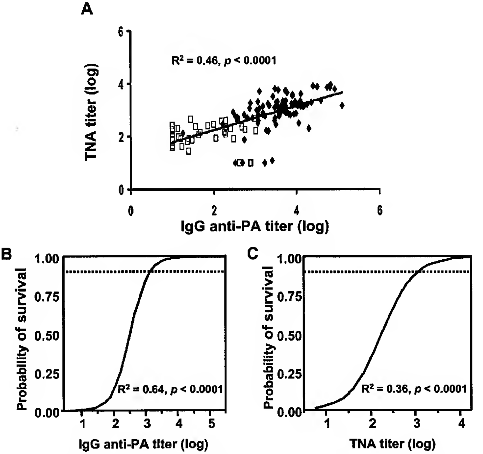

FIG. 4. Correlation between serum antibody response and survival. Mice were immunized intraperitoneally with 8 to 25 μl of AVA plus free or PLG-adsorbed ODN. Two weeks postimmunization, serum IgG anti-PA and toxin-neutralizing antibody titers were determined, and the mice were challenged intraperitoneally with 9×10^3 50% lethal doses of STI spores. Results from four independent experiments involving a total of 130 mice are shown. (A) Linear regression of IgG anti-PA versus toxin-neutralizing antibody titers in mice that succumbed to (□) or survived (♦) infection. (B) Logistic regression of survival versus IgG anti-PA titer. (C) Logistic regression of survival versus toxin-neutralizing antibody titer. TNA, toxin-neutralizing activity.

of the resultant anti-PA response was high, as evidenced by enhanced toxin-neutralizing activity, improved in vivo protection, and high levels of IgG2a antibody (known to promote the clearance of bacterial infection by complement-mediated cytotoxicity) (29).

CpG ODN and PLG microparticles represent distinct forms of vaccine adjuvant. CpG ODN interact with immune cells expressing Toll-like receptor 9 (including B cells and plasmacytoid dendritic cells), stimulating them to secrete proinflammatory and Th1 cytokines and chemokines and to undergo functional maturation (20). CpG ODN have been shown to improve the immune response to coadministered vaccines, including AVA (5, 10, 20, 22). Clinical trials indicate that CpG ODN are safe and effective in humans, significantly improving the antibody response elicited by a coadministered hepatitis B vaccine (11).

PLG microparticles are composed of the same biodegradable and biocompatible polymers used safely in adsorbable stitches for many years (38). Antigen can be adsorbed onto (or encapsulated within) PLG microparticles. The antigen-bound particles are readily taken up and processed by professional antigen-presenting cells, promoting the induction of humoral and cytotoxic T-lymphocyte responses (7, 34, 35, 38, 39). Since the adjuvant-like properties of PLG microparticles differ from those of CpG ODN, Singh et al. examined whether the combination could improve the immunogenicity of a coadministered antigen (37). Using $p55^{gag}$, a weak antigen, they showed that CpG ODN-coated PLG microparticles significantly boosted the cytotoxic T-lymphocyte response elicited compared to antigen alone or combined with free CpG ODN or PLG (37). The current results extend that finding by demonstrating that, when combined with a strong immunogen such as AVA, CpG ODN-PLG boost and accelerate the resultant immune response. This response persisted at protective levels throughout the 4-month duration of the experiment (Fig. 1 and 4). The absolute duration of protection, and whether CpG ODN-PLG can boost the secondary immune response elicited by AVA are subjects of ongoing investigation.

A goal of anthrax vaccine developers is to reduce the frequency and severity of adverse reactions. The results indicate that CpG ODN-PLG had an antigen-sparing effect, reducing the amount of AVA required to elicit protection by >20-fold. By reducing antigen load, CpG ODN-PLG might lessen the risk of adverse reactions to an AVA-based vaccine. Yet a recent report suggests that daily administration of high-dose CpG ODN for up to 3 weeks causes severe toxicity (14). Fortunately, neither local nor systemic reactions were observed in mice vaccinated with CpG ODN-PLG plus AVA in the current study. This is consistent with previous work showing that modest doses of CpG ODN can be used safely as vaccine adjuvants (4, 5, 11, 20, 21, 22, 37).

Both IgG anti-PA and toxin-neutralizing activity have been proposed as surrogate markers for vaccine efficacy, although their relative merit is uncertain (27). Resolving this issue is of considerable importance, as the decision to license future anthrax vaccines will rely on surrogate markers of protection (since conventional phase III efficacy studies cannot be conducted with biothreat pathogens). While IgG anti-PA and toxin-neutralizing antibody levels both correlated with survival, two-parameter logistic regression analysis showed the former to have greater sensitivity and specificity in predicting survival (Fig. 4). From the perspective of evaluating the likely efficacy of a vaccination campaign, serum IgG anti-PA antibody titer would be useful in predicting survival following defined levels of pathogen exposure (Fig. 4).

Current findings demonstrate that CpG ODN-PLG can increase the speed and magnitude of the humoral immune response induced by AVA. This vaccine-adjuvant combination elicited protective immunity within 1 week, suggesting that a CpG ODN-PLG-based vaccine might accelerate the induction of a protective immune response in individuals potentially exposed to anthrax. Such a vaccine might also be administered to emergency personnel prior to deployment to a site of anthrax release. The current results also help clarify the relationship between serum anti-PA and toxin-neutralizing antibody titers and their utility as surrogate markers for protection. Ongoing research suggests that CpG ODN-PLG also boost the response of vaccine candidates targeting other biowarfare pathogens. While such studies need to be repeated in appropriate nonhuman primate challenge models, they suggest that CpG ODN-PLG may be useful as adjuvants for a broad range of novel vaccines.

ACKNOWLEDGMENTS

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The assertions herein are the private ones of the authors and are not to be construed as official or as reflecting the views of USAMRIID or the FDA at large.

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REFERENCES

1. **Agresti, A., and B. A. Coull.** 1996. Order-restricted tests for stratified comparisons of binomial proportions. Biometrics 52:1103–1111.
2. **Ballas, Z. D., W. L. Rasmussen, and A. M. Krieg.** 1996. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J. Immunol. 157:1840–1847.
3. **Briones, M., M. Singh, M. Ugozzoli, J. Kazzaz, S. Klakamp, G. Ott, and D. O'Hagan.** 2001. The preparation, characterization, and evaluation of cationic microparticles for DNA vaccine delivery. Pharm. Res 18:709–712.
4. **Davis, H. L., I. Suparto, R. Weeratna, Jumintarto, D. Iskandriati, S. Chamzah, A. Ma'ruf, C. Nente, A. M. Krieg, Heriyanto, W. Smits, and D. Sajuthi.** 2000. CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. Vaccine 18:1920–1924.
5. **Davis, H. L., R. Weeranta, T. J. Waldschmidt, L. Tygrett, J. Schorr, and A. M. Krieg.** 1998. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J. Immunol. 160:870–876.
6. **Denis-Mize, K. S., M. Dupuis, M. L. MacKichan, M. Singh, B. Doe, D. O'Hagan, J. B. Ulmer, J. J. Donnelly, D. M. McDonald, and G. Ott.** 2000. Plasmid DNA adsorbed onto cationic microparticles mediates target gene expression and antigen presentation by dendritic cells. Gene Ther. 7:2105–2112.
7. **Denis-Mize, K. S., M. Dupuis, M. Singh, C. Woo, M. Ugozzoli, D. T. O'Hagan, J. J. Donnelly III, G. Ott, and D. M. McDonald.** 2003. Mechanisms of increased immunogenicity for DNA-based vaccines adsorbed onto cationic microparticles. Cell. Immunol. 225:12–20.
8. **Friedlander, A. M., and P. S. Brachman.** 1998. Anthrax, p. 729–739. *In* S. A. Plotkin and E. A. Mortimer (ed.), Vaccines. W. B. Saunders, Philadelphia, Pa.
9. **Geier, D. A., and M. R. Geier.** 2002. Anthrax vaccination and joint related adverse reactions in light of biological warfare scenarios. Clin. Exp. Rheumatol. 20:217–220.
10. **Gursel, I., M. Gursel, K. J. Ishii, and D. M. Klinman.** 2001. Sterically stabilized cationic liposomes improve the uptake and immunostimulatory activity of CpG oligonucleotides. J. Immunol. 167:3324–3328.
11. **Halperin, S. A., G. Van Nest, B. Smith, S. Abtahi, H. Whiley, and J. J. Eiden.** 2003. A phase I study of the safety and immunogenicity of recombinant hepatitis B surface antigen co-administered with an immunostimulatory phosphorothioate oligonucleotide adjuvant. Vaccine 21:2461–2467.
12. **Halpern, M. D., R. J. Kurlander, and D. S. Pisetsky.** 1996. Bacterial DNA

induces murine interferon-gamma production by stimulation of IL-12 and tumor necrosis factor-alpha. Cell. Immunol. 167:72–78.

13. **Hanna, P.** 1998. Anthrax pathogenesis and host response. Curr. Top. Microbiol. Immunol. 225:13–35.
14. **Heikenwalder, M., M. Polymenidou, T. Junt, C. Sigurdson, H. Wagner, S. Akira, R. Zinkernagel, and A. Aguzzi.** 2004. Lymphoid follicle destruction and immunosuppression after repeated CpG oligodeoxynucleotide administration. Nat. Med. 10:187–192.
15. **Ivins, B. E., M. L. Pitt, P. F. Fellows, J. W. Farchaus, G. E. Benner, D. M. Waag, S. F. Little, G. W. Anderson, Jr., P. H. Gibbs, and A. M. Friedlander.** 1998. Comparative efficacy of experimental anthrax vaccine candidates against inhalation anthrax in rhesus macaques. Vaccine 16:1141–1148.
16. **Ivins, B. E., and S. L. Welkos.** 1988. Recent advances in the development of an improved, human anthrax vaccine. Eur. J. Epidemiol. 4:12–19.
17. **Ivins, B. E., S. L. Welkos, G. B. Knudson, and S. F. Little.** 1990. Immunization against anthrax with aromatic compound-dependent (Aro-) mutants of *Bacillus anthracis* and with recombinant strains of *Bacillus subtilis* that produce anthrax protective antigen. Infect. Immun. 58:303–308.
18. **Ivins, B. E., S. L. Welkos, S. F. Little, M. H. Crumrine, and G. O. Nelson.** 1992. Immunization against anthrax with *Bacillus anthracis* protective antigen combined with adjuvants. Infect Immun. 60:662–668.
19. **Jones, T. R., N. Obaldia, R. A. Gramzinski, Y. Charoenvit, N. Kolodny, S. Kitov, H. L. Davis, A. M. Krieg, and S. L. Hoffman.** 1999. Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenic vaccine in Aotus monkeys. Vaccine 17:3065–3071.
20. **Klinman, D. M.** 2004. Immunotherapeutic uses of CpG oligodeoxynucleotides. Nat. Rev. Immunol. 4:249–258.
21. **Klinman, D. M., H. Xie, S. Little, D. Currie, and B. Ivins.** 2004. CpG oligonucleotides improve the protective immune response induced by the anthrax vaccination of rhesus macaques. Vaccine 22:2881–2886.
22. **Klinman, D. M., K. M. Barnhart, and J. Conover.** 1999. CpG motifs as immune adjuvants. Vaccine 17:19–25.
23. **Klinman, D. M., A. Yi, S. L. Beaucage, J. Conover, and A. M. Krieg.** 1996. CpG motifs expressed by bacterial DNA rapidly induce lymphocytes to secrete IL-6, IL-12 and IFNg. Proc. Natl. Acad. Sci. USA 93:2879–2883.
24. **Krieg, A. M., A. Yi, S. Matson, T. J. Waldschmidt, G. A. Bishop, R. Teasdale, G. A. Koretzky, and D. M. Klinman.** 1995. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 374:546–548.
25. **Lane, H. C., J. L. Montagne, and A. S. Fauci.** 2001. Bioterrorism: a clear and present danger. Nat. Med. 7:1271–1273.
26. **Little, S. F., and B. E. Ivins.** 1999. Molecular pathogenesis of *Bacillus anthracis* infection. Microbes Infect. 1:131–139.
27. **Little, S. F., B. E. Ivins, P. F. Fellows, M. L. Pitt, S. L. Norris, and G. P. Andrews.** 2004. Defining a serological correlate of protection in rabbits for a recombinant anthrax vaccine. Vaccine 22:422–430.
28. **O'Hagan, D., M. Singh, M. Ugozzoli, C. Wild, S. Barnett, M. Chen, M. Schaefer, B. Doe, G. R. Otten, and J. B. Ulmer.** 2001. Induction of potent immune responses by cationic microparticles with adsorbed human immunodeficiency virus DNA vaccines. J. Virol. 75:9037–9043.
29. **Oishi, K., N. L. Koles, G. Guelde, and M. Pollack.** 1992. Antibacterial and protective properties of monoclonal antibodies reactive with *Escherichia coli* O111:B4 lipopolysaccharide: relation to antibody isotype and complement-fixing activity. J. Infect. Dis. 165:34–45.
30. **Pittman, P. R., P. H. Gibbs, T. L. Cannon, and A. M. Friedlander.** 2001. Anthrax vaccine: short-term safety experience in humans. Vaccine 20:972–978.
31. **Pittman, P. R., G. Kim-Ahn, D. Y. Pifat, K. Coonan, P. Gibbs, S. Little, J. G. Pace-Templeton, R. Myers, G. W. Parker, and A. M. Friedlander.** 2002. Anthrax vaccine: immunogenicity and safety of a dose-reduction, route-change comparison study in humans. Vaccine 20:1412–1420.
32. **Quinn, C. P., and P. C. Turnbull.** 1998 Anthrax, p. 799–818. *In* M. Ballow and M. Sussman (ed.), Topley and Wilson's microbiology and microbial infections Collier, London, England.
33. **Ready, T.** 2004. US soldiers refuse to fall in line with anthrax vaccination scheme. Nat. Med. 10:112.
34. **Sanceau, J., T. Kaisho, T. Hirano, and J. Wietzerbin.** 1995. Triggering of the human interleukin-6 gene by interferon-gamma and tumor necrosis factor-alpha in monocytic cells involves cooperation between interferon regulatory factor-1, NF-kB, and Sp1 transcription factors. J. Biol. Chem. 270:27920–27931.
35. **Singh, M., J. Kazzaz, M. Ugozzoli, J. Chesko, and D. T. O'Hagan.** 2004. Charged polylactide co-glycolide microparticles as antigen delivery systems. Expert Opin. Biol Ther 4:483–491.
36. **Singh, M., X. M. Li, J. P. McGee, T. Zamb, W. Koff, C. Y. Wang, and D. T. O'Hagan.** 1997. Controlled release microparticles as a single dose hepatitis B vaccine: evaluation of immunogenicity in mice. Vaccine 15:475–481.
37. **Singh, M., G. Ott, J. Kazzaz, M. Ugozzoli, M. Briones, J. Donnelly, and D. T. O'Hagan.** 2001. Cationic microparticles are an effective delivery system for immune stimulatory CpG DNA. Pharm. Res 18:1476–1479.
38. **Vajdy, M., and D. T. O'Hagan.** 2001. Microparticles for intranasal immunization. Adv. Drug Deliv. Rev 51:127–141.
39. **Valiante, N. M., D. T. O'Hagan, and J. B. Ulmer.** 2003. Innate immunity and biodefence vaccines. Cell. Microbiol 5:755–760.
40. **Verthelyi, D., R. T. Kenney, R. A. Seder, A. A. Gam, B. Friedag, and D. M. Klinman.** 2002. CpG oligodeoxynucleotides as vaccine adjuvants in primates. J. Immunol. 168:1659–1663.
41. **Welkos, S., S. Little, A. Friedlander, D. Fritz, and P. Fellows.** 2001. The role of antibodies to *Bacillus anthracis* and anthrax toxin components in inhibiting the early stages of infection by anthrax spores. Microbiology 147:1677–1685.
42. **Welkos, S. L., and A. M. Friedlander.** 1988. Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. Microb. Pathog. 5:127–139.

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CpG oligonucleotides improve the protective immune response induced by the anthrax vaccination of rhesus macaques

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Abstract

Synthetic oligodeoxynucleotides (ODN) containing unmethylated CpG motifs act as immune adjuvants, improving the immune response elicited by co-administered vaccines. Combining CpG ODN with anthrax vaccine adsorbed (AVA, the licensed human vaccine) increased the speed, magnitude and avidity of the resultant anti-anthrax response. The protective activity of these Abs was established by passive transfer to anthrax-challenged mice. The ability of CpG ODN to accelerate and magnify the immune response to AVA suggests this strategy may contribute to the development of prophylactic and therapeutic vaccines against biothreat pathogens.
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Keywords: CpG oligonucleotide; Anthrax; Vaccine adjuvant

1. Introduction

Anthrax is caused by the gram-positive bacterium *Bacillus anthracis* [1,2]. Anthrax spores are highly resistant to environmental degradation and can remain infectious for decades. In the past, human exposure to anthrax resulted primarily from cutaneous contact with infected livestock [1,2]. Recently, spores formulated to readily infect the respiratory tract were intentionally released by bioterrorists in the US [3]. This caused immediate morbidity and mortality, and raised the specter of future exposure to these long-lived spores [3].

Vaccination is the least costly and most effective method of reducing susceptibility to anthrax infection and accelerating the development of protective immunity following pathogen exposure [1]. Neutralizing Abs directed against the bacteria's "protective antigen" (PA) reduce pathogenicity by preventing anthrax toxin from binding to host cells, inhibiting the germination of spores, and improving the phagocytosis/killing of spores by macrophages [4–7]. Unfortunately, the currently licensed human anthrax vaccine adsorbed (AVA) requires six vaccinations over 18 months followed by yearly boosters to induce and maintain protective IgG anti-PA titers [8,9]. In some vaccinees, this immunization regimen causes undesirable side effects [9].

Synthetic oligodeoxynucleotides (ODN) containing immunostimulatory "CpG motifs" can improve the immune response to co-administered antigens [10–12]. CpG ODN interact with Toll-like receptor 9 expressed by B cells and plasmacytoid dendritic cells [13–16], improving antigen presentation and triggering the production of chemokines and Th1 and pro-inflammatory cytokines (including IFNγ, IL-6, IL-12, IL-18 and TNFα) [13,14,17,18]. In mice, CpG ODN have been shown to boost the protective efficacy of vaccines against bacterial, viral and parasitic pathogens [19–23]. However, due to evolutionary divergence in CpG recognition between species, ODN that are highly active in rodents may be poorly immunostimulatory in primates [24–26]. Thus, pre-clinical studies to examine whether CpG ODN can accelerate and boost the immune response elictied by AVA must be conducted in a relevant primate model. This work provides evidence that co-administering GMP-grade CpG ODN with AVA to rhesus macaques increases the rapidity, titer, affinity, and protective efficacy of their resultant IgG anti-PA response.

Abbreviations: rPA, recombinant PA antigen; PA, protective antigen; AVA, anthrax vaccine adsorbed (licensed human anthrax vaccine); ODN, oligodeoxynucleotide

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EXHIBIT B

2. Materials and methods

2.1. Reagents

The phosphorothioate ODN mixture containing equimolar amounts of ATCGACTCTCGAGCGTTCTC, TCGTTCG TTCTC and TCGAGCGTTCTC was synthesized at the Center for Biologics Core Facility. ODN 7909 was provided by Coley Pharmaceuticals. All ODN had less than <0.1 EU of endotoxin/mg of DNA as assessed by a Limulus amebocyte lysate assay (QCL-1000, BioWhittaker). AVA was obtained from BioPort Corporation (East Lansing, MI).

The attenuated non-encapsulated Sterne strain of anthrax was obtained from the culture collection of the United States Army Medical Research Institute of Infectious Diseases, Fort Detrick, MD. Spores were prepared and stored as previously described [27].

2.2. Animals

All animal studies were ACUC approved and were conducted in AAALAC accredited facilities. Animals were monitored daily by veterinarians. Specific pathogen-free A/J mice were obtained from the Jackson Laboratories (Bar Harbor, ME) and housed in sterile micro-isolator cages in a barrier environment.

In the first experiment, rhesus macaques (3–5 kg, $N = 6$ per group) were immunized s.c. at 0 and 6 weeks with 0.5 ml of AVA plus 250 μg of an equimolar mixture of 3 CpG ODN (described below), and then "challenged" with 10^5 Sterne strain anthrax spores when serum anti-PA titers returned to baseline at week 26. Serum Ab levels were monitored 3 weeks post-immunization and 2 weeks post-boost per challenge. In the second experiment, macaques (3–5 kg, $N = 5$ per group) were immunized s.c. with 0.5 ml of AVA plus 500 ug of ODN 7909 or the same mixture of 3 CpG ODN mentioned above. Animals were bled at least weekly for 6 weeks. All treatments were administered and peripheral blood samples obtained from ketamine anesthetized animals (10 mg/kg, Ketaject, Phoenix Pharmaceuticals, St. Joseph, MD).

2.3. Anti-PA ELISA and avidity assays

Anti-PA titers were measured by coating 96-well Immulon 2 microtiter plates (Thermo LabSystems, Franklin MA) with 1 μg/ml of recombinant PA (rPA) (produced at USAMRIID as previously described [28]). Serum samples were serially diluted in PBS plus 5% non-fat dry milk and incubated on these plates for 1 h at RT. The plates were washed, and for avidity assays overlaid for 15 min with 200 μl of 6 M urea. Bound Ab was detected using peroxidase-conjugated goat anti-human IgG (Kirkegaard & Perry, Gaithersburg, MD) followed by ABTS substrate (Kirkegaard & Perry). Absorbance values were measured at 405 nm, and Ab titers calculated from the linear portion of the titration curve containing at least three contiguous dilutions ($R^2 > 0.9800$). Ab titers represent the reciprocal of the dilution that resulted in an absorbance value of 0.400, which was approximately twice background levels. For avidity comparisons, titers were determined by comparison to a standard curve generated using high-titered anti-PA serum. All samples were analyzed in triplicate.

2.4. Serum transfer study

Serum from all monkeys in each treatment group was pooled. One hundred microliters of pooled serum was injected i.p. into 6-week-old male A/J mice ($N = 20$ per group). The following day, mice were challenged i.p. with 500 μl of PBS containing 30 LD_{50} Sterne strain anthrax spores. Mice were monitored daily for 2 weeks, and time to death was recorded.

2.5. Toxin neutralization assay

RAW264.7 cells were plated overnight in 96-well microtiter plates at a concentration of 30,000 cells per well. Serum from vaccinated monkeys was serially diluted in complete medium (starting at 1:20) and incubated with 100 ng/ml rPA plus 100 ng/ml lethal factor (Research Diagnostics Inc., Flanders NJ) for 1 h at 37 °C. This mixture was added to the cells for 4 h at 37 °C. Ten microliters of MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide, 5 mg/ml in PBS, Sigma, St. Louis MO) was added for 2 h, followed by lysis buffer (25 g SDS in 50% DMF, pH 4.7) to dissolve the formazan crystals. Absorbance values were read at 570 with a reference filter of 690.

2.6. Statistical analysis

Differences in serum anti-PA Ab titers were evaluated by multiple regression ANOVA. Differences in survival were evaluated using Chi-square analysis of Kaplan–Meier curves.

3. Results

3.1. CpG ODN increase the speed and titer of the IgG anti-PA Ab response induced by AVA immunization

Initial studies examined the effect of co-administering a mixture of CpG ODN [29] with AVA on the resultant immune response. The production of serum IgG anti-PA Abs was monitored, since such Abs are necessary and sufficient to protect against anthrax infection [1]. As seen in Fig. 1, adding CpG ODN to AVA increased the primary and secondary IgG response by two- to three-fold when compared to AVA alone ($P < 0.02$). At week 26, when Ab titers had

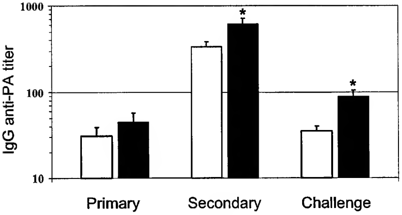

Fig. 1. Rhesus macaques (6 per group) were immunized s.c. with 0.5 ml of AVA alone (□) or combined with 250 ug of a mixture of the 3 CpG ODN described in Section 2 (■). Animals were boosted with the same material at week 6, and challenged with 10^5 Sterne strain anthrax spores at week 26. IgG anti-PA titers were measured in the serum of each animal 3 weeks post-immunization and 2 weeks post-boost per challenge. Results represent the mean ± S.E.M. of these titers. (*) Significantly higher IgG anti-PA titer at that time point, compared to animals immunized with AVA alone, $P < 0.05$.

fallen to near baseline levels, the animals were challenged with the attenuated Sterne strain of anthrax. The resultant IgG anti-PA response was significantly higher in macaques primed and boosted with AVA plus CpG ODN ($P < 0.01$).

The quality of a vaccine-adjuvant combination is reflected by both the avidity and titer of the Abs induced. To assess the avidity of the IgG anti-PA Abs, their ability to remain bound to PA in the presence of 6 M urea was evaluated [30,31]. As expected, affinity-matured Abs induced by secondary immunization were significantly more avid than those elicited by primary immunization (Fig. 2, $P < 0.01$). The avidity of the secondary anti-PA response of macaques immunized with AVA + CpG ODN was significantly higher than that of animals immunized with AVA alone ($P < 0.01$).

Based on this evidence that CpG ODN could improve the immunogenicity of AVA, a second experiment was conducted utilizing GMP-grade CpG ODN optimized for human use. Clinical grade CpG ODN 7909 (currently undergoing clinical trials for cancer therapy), the original CpG ODN mixture, or AVA alone, was administered to rhesus macaques. As seen in Fig. 3, the CpG ODN mixture again induced a stronger immune response than AVA alone. However, ODN 7909 triggered an even higher IgG anti-PA response. Starting by 11 days post-vaccination, ODN 7909 plus AVA was significantly more immunogenic than AVA alone ($P < 0.05$), generating a >3-fold higher IgG anti-PA response over the first month ($P < 0.01$).

3.2. *Protective efficacy of the IgG anti-PA response*

The critical measure of an antigen-adjuvant combination is its ability to induce protective immunity. Due to restrictions on the use of macaques in lethal anthrax challenge experiments, several alternative approaches were used to examine whether protective immunity was elicited by vaccination with AVA plus CpG ODN. In the first experiment,

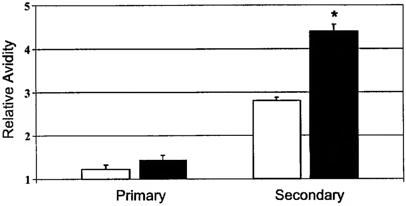

Fig. 2. The relative avidity ± S.E.M. of the IgG anti-PA Abs in the serum of animals immunized and boosted as described in Fig. 1 was determined by elution with 6 M urea. (*) Significantly greater avidity compared to animals immunized with AVA alone, $P < 0.03$.

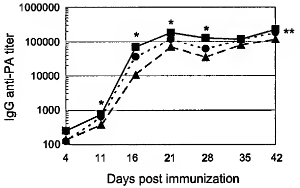

Fig. 3. Rhesus macaques (5 per group) were immunized s.c. with 0.5 ml of AVA alone (▲) or combined with 500 ug of the mixture of CpG ODN used in Fig. 1 (●) or ODN 7909 (■). IgG anti-PA titers were measured by ELISA in the serum of each animal at multiple time points post-immunization. Results represent the mean ± S.E.M. of these titers. (*) Significantly higher IgG anti-PA titer at that time point, compared to animals immunized with AVA alone, $P < 0.05$. (**) Significantly higher cumulative IgG anti-PA titer compared to animals immunized with AVA alone, $P < 0.01$.

immunized macaques were "challenged" with the attenuated (non-lethal) Sterne strain of *B. anthracis*. Challenged animals immunized with AVA plus CpG ODN mounted a 3-fold stronger immune response than animals immunized with AVA alone (Fig. 1, $P < 0.03$).

Second, the ability of serum Abs from immunized macaques to passively protect mice from anthrax challenge was explored. A/J mice are susceptible to the attenuated Sterne strain of anthrax, but are protected by neutralizing anti-PA Abs. Serum from pre-immune or immunized macaques was passively transferred to recipient mice (20 per group). As seen in Fig. 4, animals that received pre-immune serum or serum from AVA immunized macaques rapidly succumbed to challenge by 30 LD_{50} Sterne strain *B. Anthracis* spores. In contrast, serum from macaques immunized with AVA plus CpG ODN protected nearly half of recipient mice from lethal challenge ($P < 0.03$).

Table 1
Passive transfer of protection to naive recipient mice

Treatment	Donor	Recipient	Protection (%)
Serum neutralizing titer			
Pre-immunization	0 ± 0	10 ± 5	10
AVA alone	25 ± 5	43 ± 33	10
AVA + CpG ODN	434 ± 109*	2510 ± 1150*	45*

Rhesus macaques were immunized s.c. with 0.5 ml of AVA alone or combined with 500 μg of ODN 7909. Pre-immune serum, or serum 11 days post-vaccination, was pooled and injected i.p. into A/J mice (0.1 ml per recipient). The following day, mice were bled and then challenged with 30 LD_{50} of Sterne strain anthrax. The serum neutralizing titer of both donors (5 per group) and recipients ($N = 20$), and percent of recipients surviving challenge, are shown. Please note that mouse serum gave a higher background than macaque serum in this neutralizing assay.
* Significantly higher than AVA group, $P < 0.05$.

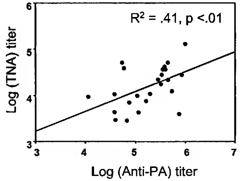

Fig. 5. IgG anti-PA titers and toxin neutralizing titers were monitored in sera from macaques immunized with AVA alone or AVA plus CpG ODN. Each data point represents the titer of a single serum measured by each assay.

These differences in susceptibility correlated with the titer of toxin neutralizing Abs present in donor serum transferred to recipient mice. Macaques immunized with AVA + ODN 7909 had on average a 17-fold higher toxin neutralizing titer than those immunized with AVA alone (Table 1, $P < 0.03$).

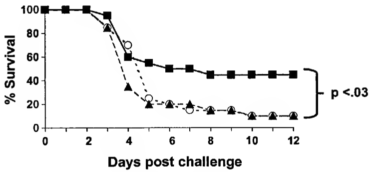

Fig. 4. Pre-immune serum (○), or serum from rhesus macaques vaccinated 11 days earlier with AVA alone (▲) or AVA + CpG ODN 7909 (■) was pooled and injected i.p. into A/J mice (0.1 ml per recipient). The following day, mice were challenged with 30 LD_{50} of Sterne strain anthrax. Results of two independent experiments involving at combined total of 20 recipients per treatment are shown.

A similar difference in neutralizing Ab levels was detected in the serum of recipient mice immediately prior to challenge (Table 1, $P < 0.05$). As expected, serum IgG anti-PA titers correlated closely with toxin neutralizing activity (Fig. 5, $R^2 = 0.41$, $P < 0.01$).

4. Discussion

CpG motifs present in bacterial DNA and synthetic oligonucleotides directly stimulate B cells and plasmacytoid dendritic cells that express Toll-like receptor 9 [15,16]. This initiates an immunostimulatory cascade that results in the functional maturation of professional antigen presenting cells and the production of Th1 and pro-inflammatory cytokines and chemokines [13,14,32,33].

Studies in mice demonstrated that CpG ODN could serve as immune adjuvants, significantly improving the immune response elicited by protein antigens and vaccines [21,22,34,35]. CpG ODN boosted the immune response elicited by vaccines against influenza, measles, hepatitis B surface Ag and tetanus toxoid by one to three orders of magnitude, while increasing the production of Th1 cytokines and the activity of antigen-specific CTL [34–39]. Due to evolutionary divergence in TLR 9 expression between species, CpG motifs optimized for activity in humans are less effective in mice [24–26]. Thus, pre-clinical studies of CpG ODN planned for human use are best performed in non-human primates [12,24–26,28]. Studies of orangutans, aotus monkeys, and rhesus macaques showed that CpG ODN boosted the immune response elicited by the hepatitis B vaccine and heat-killed leishmania vaccine by several fold [10–12].

The current work evaluated whether clinical grade CpG ODN (developed for use in cancer immunotherapy) could improve the immune response elicited by AVA (the licensed human anthrax vaccine). Results indicate that macaques immunized with AVA plus CpG ODN mounted a stronger immune response when challenged with Sterne strain anthrax spores (Fig. 1). The combination of CpG ODN plus AVA triggered a faster, higher avidity, and higher-titered immune response than vaccine alone, resulting in a significant improvement in protective immunity against anthrax (Fig. 4).

The induction of IgG anti-PA Abs is the most relevant measure of vaccine immunogenicity, since these Abs confer protection against infection [1]. Consistent with previous studies, serum levels of IgG anti-PA Abs correlated closely with toxin neutralizing activity, a surrogate marker for protective efficacy [1]. While antigen-specific T cell and cytokine responses may also be affected by CpG ODN [14,40], reagents to monitor anthrax-specific cellular responses in macaques are not available. Yet preliminary studies in mice showed that adding CpG ODN to AVA stimulated a predominantly Th1-biased immune response characterized by increased levels of IgG2a anti-PA antibodies (data not shown).

Current results demonstrate that the avidity of the secondary IgG anti-PA immune response was significantly improved by inclusion of CpG ODN (Fig. 2). This effect is consistent with the documented ability of CpG ODN to promote the functional maturation of professional APC [41]. In this context, ongoing studies suggest that other methods of targeting AVA to professional APC also results in higher-titered, more avid immune responses.

As with all novel therapies, the possibility of adverse side effects was considered. In previous studies, CpG ODN were safely administered to rodents and primates without adverse consequences [12,42]. In the current work, no serious local or systemic adverse reactions were observed in any of the macaques treated with CpG ODN plus AVA.

Vaccines targeting biothreat pathogens are typically designed for prophylactic (pre-exposure) use. However, vaccines capable of accelerating the development of protective immunity can be of therapeutic benefit to individuals exposed to biothreat pathogens (e.g., workers in anthrax-contaminated buildings) and/or for "ring vaccination" of individuals at sites of known infection. For these latter purposes, the capacity of CpG ODN to accelerate as well as increase the titer of anti-anthrax Abs is of particular interest. Our results demonstrate that co-administering CpG ODN with AVA generates high levels of toxin neutralizing antibodies very rapidly (exceeding AVA alone by 17-fold at 11 days post-immunization). Passive transfer of these serum antibodies protected nearly half of naive mice from challenge with 30 LD_{50} of anthrax spores (Fig. 4, $P < 0.03$ versus AVA alone). These findings support the further development of CpG ODN as an adjuvant for vaccines targeting biothreat pathogens.

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References

[1] Friedlander AM, Brachman PS. Anthrax. In: Plotkin SA, Mortimer EA, editors. Vaccines. Philadelphia (PA): WB Saunders; 1998. p. 729–39.

[2] Quinn CP, Turnbull PC. Anthrax. In: Ballow M, Sussman M, editors. Topley and Wilson's microbiology and microbial infections, 98 A.D. London: Collier. p. 799–818.

[3] Lane HC, Montagne JL, Fauci AS. Bioterrorism: a clear and present danger. Nat Med 2001;7(12):1271–3.

[4] Ivins BE, Welkos SL, Liffle SF, Crumrine MH, Nelson GO. Immunization against anthrax with *Bacilus anthracis* protective antigen combined with adjuvants. Infect Immun 1992;60:662–8.

[5] Welkos SL, Friedlander AM. Comparative safety and efficacy against *Bacillus anthracis* of protective antigen and live vaccines in mice. Microb Pathog 1988;5(2):127–39.
[6] Little SF, Ivins BE. Molecular pathogenesis of *Bacillus anthracis* infection. Microb Infect 1999;1(2):131–9.
[7] Welkos S, Little S, Friedlander A, Fritz D, Fellows P. The role of antibodies to *Bacillus anthracis* and anthrax toxin components in inhibiting the early stages of infection by anthrax spores. Microbiology 2001;147(Pt 6):1677–85.
[8] Pittman PR, Kim-Ahn G, Pifat DY, Coonan K, Gibbs P, Little S, et al. Anthrax vaccine: immunogenicity and safety of a dose-reduction, route-change comparison study in humans. Vaccine 2002;20(9–10):1412–20.
[9] Pittman PR, Gibbs PH, Cannon TL, Friedlander AM. Anthrax vaccine: short-term safety experience in humans. Vaccine 2001;20(5–6):972–8.
[10] Davis HL, Suparto II, Weeratna RR, Jumintarto DD, Chamzah SS, Ma'ruf AA, et al. CpG DNA overcomes hyporesponsiveness to hepatitis B vaccine in orangutans. Vaccine 2000;19(4/5):413–22.
[11] Jones TR, Obaldia N, Gramzinski RA, Charoenvit Y, Kolodny N, Kitov S, et al. Synthetic oligodeoxynucleotides containing CpG motifs enhance immunogenic vaccine in Aotus monkeys. Vaccine 1999;17(23–24):3065–71.
[12] Verthelyi D, Kenney RT, Seder RA, Gam AA, Friedag B, Klinman DM. CpG oligodeoxynucleotides as vaccine adjuvants in primates. J Immunol 2002;168(4):1659–63.
[13] Krieg AM, Yi A, Matson S, Waldschmidt TJ, Bishop GA, Teasdale R, et al. CpG motifs in bacterial DNA trigger direct B-cell activation. Nature 1995;374:546–8.
[14] Klinman DM, Yi A, Beaucage SL, Conover J, Krieg AM. CpG motifs expressed by bacterial DNA rapidly induce lymphocytes to secrete IL-6, IL-12 and IFNγ. Proc Natl Acad Sci USA 1996;93:2879–83.
[15] Takeshita F, Leifer CA, Gursel I, Ishii K, Takeshita S, Gursel M, et al. Cutting Edge: role of toll-like receptor 9 in CpG DNA-induced activation of human cells. J Immunol 2001;167(7):3555–8.
[16] Hemmi H, Takeuchi O, Kawai T, Sato S, Sanjo H, Matsumoto M, et al. A Toll-like receptor recognizes bacterial DNA. Nature 2000;408:740–5.
[17] Ballas ZD, Rasmussen WL, Krieg AM. Induction of NK activity in murine and human cells by CpG motifs in oligodeoxynucleotides and bacterial DNA. J Immunol 1996;157:1840–7.
[18] Halpern MD, Kurlander RJ, Pisetsky DS. Bacterial DNA induces murine interferon-gamma production by stimulation of IL-12 and tumor necrosis factor-alpha. Cell Immunol 1996;167:72–8.
[19] Klinman DM. Therapeutic applications of CpG-containing oligodeoxynucleotides. Antisense Nucleic Acid Drug Dev 1998;8:181–4.
[20] Lipford GB, Bauer M, Blank C, Reiter R, Wagner H, Heeg K. CpG-containing synthetic oligonucleotides promote B and cytotoxic T cell responses to protein antigen: a new class of vaccine adjuvants. Eur J Immunol 1997;27:2340–4.
[21] Davis HL. Use of CpG DNA for enhancing specific immune responses. Curr Top Microbiol Immunol 2000;247:171–84.
[22] Moldoveanu Z, Homan LL, Huang WQ, Krieg AM. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. Vaccine 1999;16:1216–24.
[23] Krieg AM, Davis HL. Enhancing vaccines with immune stimulatory CpG DNA. Curr Opin Mol Ther 2001;3(1):15–24.
[24] Bauer M, Heeg K, Wagner H, Lipford GB. DNA activates human immune cells through a CpG sequence-dependent manner. Immunology 1999;97(4):699–705.
[25] Hartmann G, Krieg AM. Mechanism and function of a newly identified CpG DNA motif in human primary B cells. J Immunol 2000;164(1):944–52.
[26] Verthelyi D, Isbii KJ, Gursel M, Takeshita F, Klinman DM. Human peripheral blood cells differentially recognize and respond to two distinct CpG motifs. J Immunol 2001;166:2372–7.
[27] Ivins BE, Welkos SL, Knudson GB, Little sF. Immunization against anthrax with aromatic compound-dependent (Aro-) mutants of *Bacillus anthracis* and with recombinant strains of *Bacillus subtilis* that produce anthrax protective antigen. Infect Immun 1990;58(2):303–8.
[28] Ivins BE, Pitt ML, Fellows PF, Farchaus JW, Benner GE, Waag DM, et al. Comparative efficacy of experimental anthrax vaccine candidates against inhalation anthrax in rhesus macaques. Vaccine 1998;16(11-12):1141–8.
[29] Leifer CA, Verthelyi D, Klinman DM. Heterogeneity in the human response to immunostimulatory CpG oligodeoxynucleotides. J Immunother 2003;26(4):313–9.
[30] Eggers M, Bader U, Enders G. Combination of microneutralization and avidity assays: improved diagnosis of recent primary human cytomegalovirus infection in single serum sample of second trimester pregnancy. J Med Virol 2000;60(3):324–30.
[31] Cozon GJ, Ferrandiz J, Nebhi H, Wallon M, Peyron F. Estimation of the avidity of immunoglobulin G for routine diagnosis of chronic *Toxoplasma gondii* infection in pregnant women. Eur J Clin Microbiol Infect Dis 1998;17(1):32–6.
[32] Sparwasser T, Koch E, Vabulas RM, Heeg K, Lipford GB, Ellwart JW, et al. Bacterial DNA and immunostimulatory CpG oligonucleotides trigger maturation and activation of murine dendritic cells. Eur J Immunol 1998;28(6):2045–54.
[33] Roman M, Martin-Orozco E, Goodman JS, Nguyen M, Sato Y, Ronaghy A, et al. Immunostimulatory DNA sequences function as T helper-1 promoting adjuvants. Nat Med 1997;3:849–54.
[34] Brazolot Millan CL, Weeratna R, Krieg AM, Siegrist CA, Davis HL. CpG DNA can induce strong Th1 humoral and cell-mediated immune responses against hepatitis B surface antigen in young mice. Proc Natl Acad Sci USA 1998;95:15553–8.
[35] McCluskie MJ, Davis HL. CpG DNA is a potent enhancer of systemic and mucosal immune responses against hepatitis B surface antigen with intranasal administration to mice. J Immunol 1998;161:4463–6.
[36] Moldoveanu Z, Love-Homan L, Huang WQ, Krieg AM. CpG DNA, a novel immune enhancer for systemic and mucosal immunization with influenza virus. Vaccine 1998;16:1216–24.
[37] Branda RF, Moore AL, Lafayette AR, Mathews L, Hong R, Zon G, et al. Amplification of antibody production by phosphorothioate oligodeoxynucleotides. J Lab Clin Med 1996;128:329–38.
[38] Kovarik J, Bozzotti P, Love-Homan L, Pihlgren M, Davis HL, Lambert P-H AM, et al. CpG oligonucleotides can cirmcuvent the TH2 polorization of neonatal responses to vaccines but fail to fully redirect TH2 responses established by neonatal priming. J Immunol 1999;162:1611–7.
[39] Davis HL, Weeranta R, Waldschmidt TJ, Tygrett L, Schorr J, Krieg AM. CpG DNA is a potent enhancer of specific immunity in mice immunized with recombinant hepatitis B surface antigen. J Immunol 1998;160:870–6.
[40] Sun S, Zhang X, Tough DF, Sprent J. Type I interferon-mediated stimulation of T cells by CpG DNA. J Exp Med 1998;188:2335–42.
[41] Gursel M, Verthelyi D, Gursel I, Ishii KJ, Klinman DM. Differential and competitive activation of human immune cells by distinct classes of CpG oligodeoxynucleotides. J Leukoc Biol 2002;71:813–20.
[42] Klinman DM, Conover J, Coban C. Repeated administration of synthetic oligodeoxynucleotides expressing CpG motifs provides long-term protection against bacterial infection. Infect Immun 1999;67:5658–63.